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AMINO-NITROGEN CONTENTS OF WOOL AND COLLAGEN

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ABSTRACT

When wool, collagen, and arginine were treated with nitrous acid, increasing amounts of nitrogen were evolved with time. The continued evolution of nitrogen was due to the action of nitrous acid on the guanidine nuclei of these materials.

A new method for the determination of the arginine content of a protein is given. The method is based on the relative rates of evolution of nitrogen from the guanidine nuclei in a protein and in arginine.

Evidence is presented to show that the action of nitrous acid on the guanidine nucleus is different from its action on a free amino group. The free amino-nitrogen contents of wool and collagen were calculated by subtracting from the total nitrogen evolved that portion of nitrogen which came from the guanidine nuclei. The values obtained for the percentages of the total nitrogen as amino nitrogen are 2.53 for wool and 2.77 for collagen.

CONTENTS

I. INTRODUCTION

The free amino groups in wool and collagen have been related to the combination of these materials with acids, dyes, and tannins by various investigators. Trotman (1),[3] and Benz and Farrel (2) claimed that deamination of wool had little effect on the affinity of wool for acid dyes, but Speakman and Stott (3) later showed that the affinity of untreated wool was much higher than that of deaminated wool. Speakman and Hirst (4) attempted to account for the acid adsorbed by wool in terms of its free amino nitrogen and histidine contents, but later stated (3) that wool did not combine with acid solely through the simple amino groups. Thomas and Foster (27) worked with deaminated collagen and showed that the absence of the free amino groups greatly decreased the ability of collagen to combine with tanning material.

The method employed by all of these investigators for the determination of the free amino groups is based on the reaction between these groups and nitrous acid, whereby the nitrogen of the amino

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[3] Figures in parentheses here and throughout the text refer to the references given at the end of this paper.

group is liberated and the amino group is replaced by a hydroxyl group according to the following scheme:

$$R-\underset{\underset{COOH}{|}}{CH}-NH_2+HNO_2 \rightarrow R-\underset{\underset{COOH}{|}}{CH}-OH+H_2O+N_2 \qquad [1]$$

The values they obtained for the amino-nitrogen contents varied greatly. The results of the present investigation show that their values do not necessarily represent the true amino-nitrogen content, and were dependent on the conditions under which their determinations were made. Owing to these discrepancies their attempts to relate the free amino nitrogen to chemical combination were solely of a qualitative nature.

II. MATERIALS AND METHODS

A number of methods are now available for the determination of amino nitrogen. The advantages and limitations of each have been compared and discussed by a large number of workers (5) (6). While none of the methods is entirely satisfactory for the determination of amino nitrogen in complex mixtures, the method of Van Slyke, which is based on the reaction in equation 1 appears to be the best for estimating the free amino nitrogen in native proteins.

In this work a Van Slyke macro-apparatus (7) was used. Since the amounts of amino nitrogen obtained were small, a 3-ml gas burette was substituted for the standard 40-ml burette. The nitrous acid was obtained by the interaction of equivalent quantities of sodium-nitrite and acetic acid. The apparatus was calibrated to deliver 9.72 ml of glacial acetic acid and 38.88 ml of a 30-percent solution of sodium nitrite to the reaction vessel. After the air in the reaction vessel was displaced by nitric oxide, formed by the decomposition of the nitrous acid, all but 5 ml of the nitrous acid was forced out of the reaction vessel. The reaction vessel is calibrated to hold 20 ml of nitrous acid. However, it was found advantageous to dilute the nitrous-acid solution. The dilute solution evolved much less nitric oxide, gave more reproducible blanks, and caused less frothing. The nitrous acid remaining in the vessel was diluted with 10 ml of water, and then 10 ml of the solution to be analyzed was introduced.

At different intervals of time, the gas evolved was run into a Hempel pipette containing an alkaline permanganate solution, which absorbed the nitric oxide. The volume of nitrogen was measured and the temperature and pressure were noted. The weight of a given volume of nitrogen at a known temperature and pressure was read directly from a table prepared by Van Slyke (7). The values obtained were corrected for the amount of nitrogen obtained in blank determinations, which were made for each time interval.

The experimental errors were minimized by keeping the stopcocks well greased, changing the permanganate solutions after every five or six runs, using freshly prepared and more dilute solutions of nitrous acid, and making blank determinations under the identical conditions of the original determinations.

One of the sources of error in amino-nitrogen determinations by the Van Slyke method occurs in making a correction for the amount of nitrogen evolved by the spontaneous decomposition of the nitrous

acid itself. Since the amount of free amino nitrogen present in native proteins is very small, it is obvious that much care must be exercised in making such corrections. For a given time of reaction, the size of the blank depends on the age of the sodium-nitrite solution, and the rate of evolution of the gas. The latter, in turn, depends on the speed of shaking and on the nature of the material being analyzed. For example, suspended particles cause a more rapid evolution of gas than materials in true solution. The effects of the various factors are illustrated in table 1. The blank determinations were made both

TABLE 1.—*Effect of various factors on the volume of nitrogen obtained in blank determinations*

Blank	Time of reaction	Age of $NaNO_2$ solution	Volume of gas [1] evolved	Volume of nitrogen
			ml	ml
Water	5 min	1 hr	75	.37
Do	5 min	24 hr	75	.43
Do	5 min	3 days	75	.48
Do	5 min	7 days	75	.49
Do	5 min	7 days	125	.55
Cellulose	5 min	1 hr	75	.43
Water	15 min	1 hr	85	.42
Cellulose	15 min	1 hr	85	.46
Water	30 min	1 hr	100	.43
Cellulose	30 min	1 hr	100	.48
Water	2 hr	1 hr	125	.51
Cellulose	2 hr	1 hr	125	.53
Water	20 hr	1 hr	150	.91
Cellulose	20 hr	1 hr	150	.90

[1] The volume of gas evolved was estimated and the values are accurate to about ± 5 ml.

on 10-ml portions of distilled water and on 10-ml portions of a 1-percent suspension of powdered cellulose. The initial amounts of nitrogen evolved were higher when powdered cellulose was used than with water alone, but they became about equal after 2 hours. In the subsequent determinations on the wool and collagen suspensions, the corrections applied were obtained on blanks containing about the same weights of cellulose as of the protein used. In all determinations the reaction vessel was shaken during the last 3 minutes.

The wool used was prepared from raw stock which had been extracted with alcohol and ether and washed with water. The clean fibers were ground in a pebble mill for 48 hours and the total nitrogen of the resulting powder was determined. The values were corrected for ash and moisture.

Standard hide powder, American Leather Chemists Association 1933, prepared in the same manner as the wool, was used for the determinations on collagen.

The suspensions were prepared by shaking approximately 1-g samples of the powder (particle size 0.3 to 2.0μ) in 100 ml of water. A 10-ml aliquot of the suspension was measured in the graduated cylinder of the Van Slyke apparatus and run into a Kjehldahl flask for determination of total nitrogen. Another 10-ml aliquot was then measured in the same cylinder and run into the Van Slyke apparatus for determination of amino nitrogen.

The glycine and arginine hydrochloride were from the Eastman Kodak Co.

III. EXPERIMENTAL

While it is known that the α-amino groups of the individual amino acids react completely with nitrous acid in 5 to 10 minutes, the time required for the amino nitrogen in proteins may be much longer. As a preliminary investigation, the evolution of amino nitrogen from glycine when treated with nitrous acid for different lengths of time was determined. The rates of evolution of nitrogen from the nitrous acid alone and from the nitrous acid plus the glycine are shown in figure 1. Since the curves are parallel, it is obvious that the increasing amounts of nitrogen, obtained by prolonging the time of reaction, come from the nitrous acid itself. The amino-nitrogen content of glycine at any time is obtained by subtracting the values on the lower curve from the corresponding values on the upper curve. These results are given in table 2. The high values obtained are in accord with those found by Van Slyke (8), and Levene and Van Slyke (9),

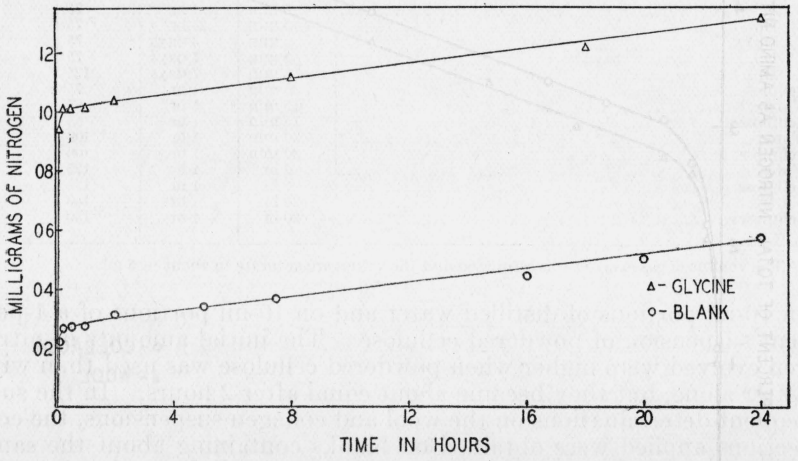


FIGURE 1.—*Relative rates of evolution of nitrogen from nitrous acid alone and from nitrous acid and glycine.*

who obtained values of 103 to 107 percent of the total nitrogen of glycine as amino nitrogen. The anomalous results appear to be independent of time and to depend on some specific reaction of the glycine or its reaction product with nitrous acid. Van Slyke's analyses of alanine gave values of 99.3 to 100.5 percent of the total nitrogen as amino nitrogen.

TABLE 2.—*Amino-nitrogen content of glycine*

Time	Total nitrogen as amino nitrogen
	Percent
5 min	99.2
15 min	105.5
30 min	103.6
1 hr	103.2
2 hr	102.5
8 hr	106.3
18 hr	105.6
24 hr	105.7

When 1-percent suspensions of powdered wool and collagen were treated with nitrous acid for different lengths of time and the necessary corrections for blank determinations applied, the values obtained were not constant as in the case of the mono-amino acids, but increased with increase in time of reaction. The results are shown in figure 2. In the case of wool, this increase in the amount of nitrogen evolved with time had been previously noted by Meunier and Rey (10), and by Speakman and Hirst (4).

Since the pH of the nitrous-acid solutions was about 4, there was little reason to believe that the increased evolution of nitrogen was due to a hydrolysis occurring during the reaction. The absence of hydrolysis was demonstrated by treating wool and collagen for periods

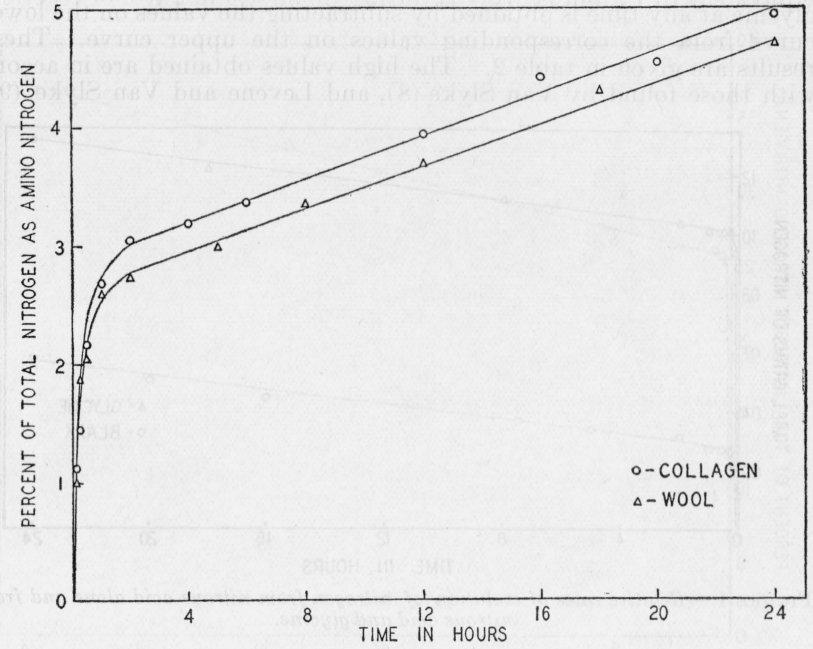


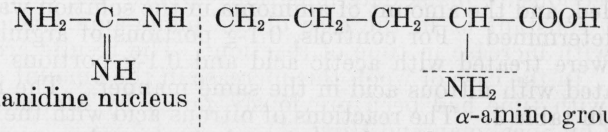
FIGURE 2.—*Rate of evolution of nitrogen from wool and collagen.*

of 24 and 48 hours with sodium-acetate-acetic-acid solutions containing acid and salt in amounts approximately equivalent to, and at about the same pH as those found in the nitrous-acid solutions. The specimens thus treated showed no measurable increase in amino-nitrogen content by the Van Slyke method.

In the absence of any hydrolysis it seemed probable that the increasing amounts of nitrogen obtained might be formed by the slow interaction of nitrous acid with a nitrogen-containing group other than an amino group.

Although the Van Slyke method has been shown to give high amino-nitrogen values for a number of the individual amino acids (8) (9), the only amino acid present in wool and collagen in appreciable amounts, which is known to give off increasing amounts of nitrogen

with time is arginine (11). Its structural formula is represented as follows:



It seemed logical, therefore, to study the rate of evolution of nitrogen from this amino acid under conditions identical with those used for wool and collagen. The results are shown in figure 3. Since arginine contains 4 nitrogen atoms, it is obvious that an amount of nitrogen equivalent to 1 atom is evolved during the first 5 minutes of reaction.

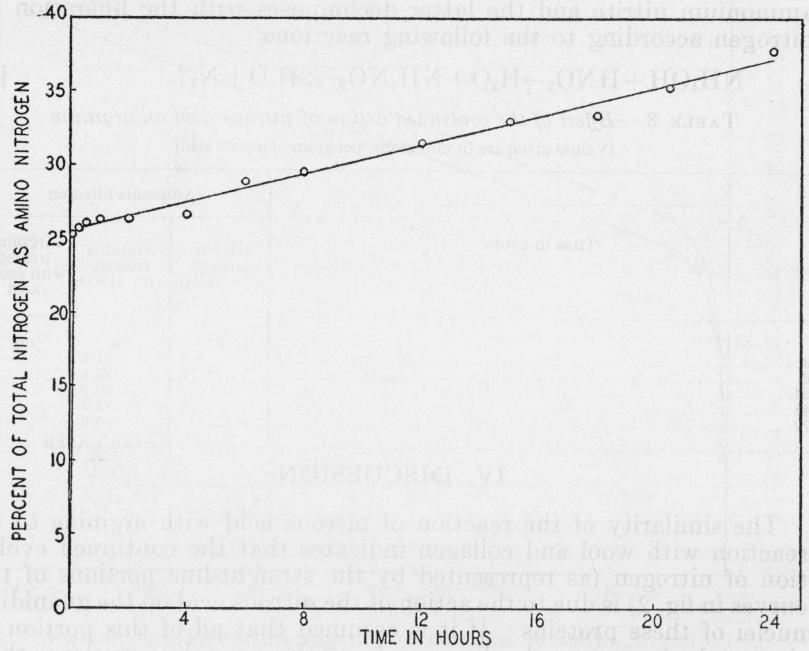


FIGURE 3.—*Rate of evolution of nitrogen from arginine.*

This nitrogen comes from the α-amino group. The nitrogen subsequently evolved is formed by the action of nitrous acid on the guanidine nucleus.

The exact structure of guanidine or the guanidine nucleus is not known, but most investigators agree that the NH_2 group, if one is present, is not a true free amino group. Their evidence will be presented later. That the nitrogen evolved by the interaction of nitrous acid with the guanidine nucleus was not produced in the same manner as nitrogen obtained by the action of nitrous acid on a free amino group was further demonstrated by the following experiments. Tenth-gram portions of arginine hydrochloride were introduced into Kjehldahl flasks and 25 ml of a nitrous-acid solution of the same concentration as used previously in the Van Slyke analyses was added to

each. After the reaction had proceeded for a definite interval of time, the solution was diluted to about 200 ml, an excess of magnesium oxide added, and the amount of ammonia in the solution was distilled off and determined. For controls, 0.1-g portions of arginine hydrochloride were treated with acetic acid and 0.1-g portions of glycine were treated with nitrous acid in the same manner. The results are recorded in table 3. The reactions of nitrous acid with the guanidine nucleus are complicated, and it is not desired to place any emphasis on these results as a possible explanation of the mechanism of the reactions. However, the data show that ammonia is a product of at least one of the reactions, and that the amount of ammonia present in the solutions reaches a maximum and then decreases with time. This is to be expected since ammonia reacts with nitrous acid to form ammonium nitrite and the latter decomposes with the liberation of nitrogen according to the following reactions:

$$NH_4OH+HNO_2\rightarrow H_2O+NH_4NO_2\rightarrow 2H_2O+N_2\uparrow \quad [2]$$

TABLE 3.—*Effect of the continued action of nitrous acid on arginine*

[Values given are in milligrams per gram of amino acid]

Time in hours	Ammonia nitrogen		
	Glycine treated with HNO_2	Arginine treated with HNO_2	Arginine treated with acetic acid
	mg	mg	mg
3	0.5	21.4	0.7
6	.6	25.1	.6
24	.8	20.3	.8
48	.9	12.3	1.2
72	----------	6.6	1.3

IV. DISCUSSION

The similarity of the reaction of nitrous acid with arginine to its reaction with wool and collagen indicates that the continued evolution of nitrogen (as represented by the straight-line portions of the curves in fig. 2) is due to the action of the nitrous acid on the guanidine nuclei of these proteins. If it is assumed that all of this portion of the total nitrogen evolved comes from the guanidine nucleus, then the fraction of the total nitrogen of the protein as arginine nitrogen is equal to the ratio of the slope of the curves for wool or collagen (fig. 2) to the slope of the curve for arginine (fig. 3). It is thus possible to calculate the arginine contents of these materials from the following equations:

$$A=\frac{(F_A)(N_P)}{(N_A)}\times 100 \quad [3]$$

where:

A =the arginine content of the protein in percent
F_A=the fraction of the total nitrogen of the protein as arginine nitrogen
N_P=the nitrogen content of the protein
N_A=the nitrogen content of arginine

$$F_A=S_P/S_A \quad [4]$$

where:

S_P=the slope of the straight line portion of the curve for proteins

S_A=the slope of the straight line portions of the curve for arginine.

Substituting equation 3 in 4

$$A=\frac{(S_P)(N_P)}{(S_A)(N_A)}\times 100 \qquad [5]$$

The slope of the curve for collagen is 0.0875, for wool 0.0865, and for arginine 0.488. The corresponding nitrogen contents are 17.40, 16.32, and 32.18 percent. Substituting these figures in equation 5, the arginine contents of the wool and collagen were found to be 9.0 and 9.7 percent, respectively.

By methods of isolation, Stewart and Rimington (12), and Vickery and Block (13) found 6.0 and 7.8 percent, respectively, of arginine in wool. Marston (14), employing indirect methods, found 10.2 percent. The arginine content of collagen has been reported as 8.2 percent by Dakin (15), and as 7.62 by Fischer, Levene, and Aders (16). These values were also obtained by isolation of the amino acid. The separation of amino acids by isolation from protein hydrolysates is a long and tedious process, and, at best, cannot be carried out without the loss of a considerable portion of the material. The summation of the amino acids of most of the proteins analyzed accounts for only about 65 to 85 percent of the protein. In view of the fact that isolation methods for amino acids are known to give low results, the method described in this paper may be considered to give better values for the arginine contents of wool and collagen.

The amino-nitrogen contents of wool and collagen were obtained by subtracting from the total nitrogen evolved that portion of the nitrogen evolved from the guanidine nucleus of the arginine in these materials. The arginine contents were those calculated in this paper. The calculations were made with the experimental values and are not points taken from the smooth curves. The results are recorded in table 4. From the average values in table 4 and the nitrogen contents of wool and collagen, the percentages of the total nitrogen as amino nitrogen were found to be 2.53 and 2.77, respectively.

TABLE 4.—*Amino nitrogen contents of wool and collagen*

[Values given are in milligrams per gram of protein]

WOOL

Time in hours	Total nitrogen evolved	Arginine nitrogen	Amino nitrogen
	mg	mg	mg
2	4.48	0.38	4.10
5	4.89	0.85	4.04
8	5.46	1.30	4.16
12	5.96	1.88	4.08
18	6.96	2.52	4.44
24	7.66	3.68	3.98
Average			4.13

TABLE 4.—*Amino nitrogen contents of wool and collogen*—Continued

COLLAGEN

Time in hours	Total nitrogen evolved	Arginine nitrogen	Amino nitrogen
	mg	mg	mg
2	5.30	0.41	4.89
4	5.56	0.53	5.03
6	5.83	1.25	4.58
12	6.88	2.03	4.85
16	7.66	2.66	5.00
20	7.92	3.22	4.70
24	8.62	3.97	4.65
Average			4.82

It is apparent from the above calculations that in this work the free amino groups have been considered to be only those that react with nitrous acid according to equation 1. Bracewell (17) suggested that other free amino groups are present in the protein molecule, but they are anomalous in that they do not react with nitrous acid. The investigations of Werner (18) showed, however, that anomalous amino groups have no real existence. Bancroft and Ridgway (19) summarized the work on the reactions of nitrous acid and guanidine. They found that various formulas have been assigned to the latter to account for its behavior, but that the experimental data did not conclusively favor any one formula. From their own investigations they concluded that guanidine and pure nitrous acid did not react, that excess acid other than nitrous acid catalyzed the reaction [4], and that the rate of evolution of nitrogen depended on the concentration of nitrous acid, the hydrogen-ion concentration, and a specific effect due to the acid itself. Clark and Gillespie (20) showed that in the presence of sodium carbonate, benzenesulphonyl chloride combined only with the α-amino group in arginine. The evidence indicates that an amino group does not exist in guanidine, or, if one does exist, it does not function as a free amino group.

Whether or not the guanidine nucleus is considered to contain a free amino group, it is obvious that treating a native protein with nitrous acid for an arbitrary length of time will not necessarily give a value which represents the true amino-nitrogen content of that protein. Meunier and Rey (10), on treating wool with nitrous acid for 7 hours, found that 5.7 mg of nitrogen was evolved from each gram of material. They assumed this to be the content of free amino nitrogen. Speakman and Hirst (4) likewise treated wool for 24 hours and obtained values of 9.2 and 11.9 mg of nitrogen per gram of wool. Subsequently, Speakman and Stott (3) studied the action of three concentrations of nitrous acid on wool and stated: "It is evident that the amino-nitrogen content of wool cannot be defined with any degree of precision. The rate of evolution of nitrogen depends to a striking extent on the concentration of nitrous acid * * *." They also determined the acid-combining capacity of untreated and deaminated wool, and found that the latter retained slightly more than half of its maximum combining capacity for

[4] Acetic acid is always present in the Van Slyke procedure. It is apparent, therefore, that the slopes of the curves will depend on the conditions under which the reactions are run.

hydrochloric acid. They concluded that the nitrogen liberated from wool could scarcely be derived solely from the arginine and lysine side-chains, and in view of a recent study of the action of nitrous acid on cystine (24) it seemed likely that part of the nitrogen was derived from the action of nitrous acid on the sulphur linkage. Lough and Lewis (24), in their studies of the action of nitrous acid on cystine, found that "extra" nitrogen was obtained only when oxidation of sulphur to sulphate occurred. Recently an attempt (25) was made in this laboratory to oxidize the sulphur in wool to sulphate with bromine and hydrochloric acid. Even with such a strong oxidizing agent only very small amounts of sulphate were obtained in periods of 24 and 48 hours. In view of this and the fact that the sulphur in wool has very different properties in general from the sulphur in cystine, it hardly seems justifiable to assume the formation of "extra" nitrogen.

The similarity of the behavior of the guanidine nucleus of the arginine in wool and collagen with that of the guanidine nucleus of the arginine itself indicates that the former is free in the native protein. Since the guanidine group is a strong base and readily combines with acids, it should be possible to account for the acid taken up by wool and collagen by their free amino groups and guanidine nuclei. Bancroft and Ridgway (19) stated that guanidine is a mono-acid base, while Schmidt, Kirk, and Appleman (21) determined the titration curve of arginine and found that arginine monohydrochloride combines with one equivalent of hydrochloric acid. On the basis of the amino groups and guanidine nuclei each combining with one equivalent of acid, the amounts of acid that will combine with wool and collagen have been calculated. The results are given in table 5. The calculated values are in good agreement with those obtained experimentally.

TABLE 5.—*Amounts of acid combined with wool and collagen*

[Values given are in milliequivalents of acid per gram of protein]

Material	Calculated	Experimental
	Milliequivalents	*Milliequivalents*
Wool	0.81	0.80 (4)
		1.0 (22)
Collagen	.90	.899 (23)
		.98 (26)

The observations of Hitchcock (23), and of Speakman and Stott (3) that deaminated gelatin and wool retain a considerable portion of their acid-combining capacities are of interest, especially since the deaminated materials were prepared under very different conditions. Gelatin was treated for 30 minutes with nitrous acid, and 5.6 mg of nitrogen, which was equivalent to slightly more than its free amino nitrogen, was removed. Wool was treated for 72 hours and about 9 mg of nitrogen was removed. This accounts for the free amino nitrogen and nearly one atom of nitrogen from the guanidine nucleus. The acid-combining capacities of the deaminated proteins are given in table 6. The results indicate that the removal of a nitrogen atom from the guanidine nucleus has not appreciably altered the latter's

basic properties. The evidence suggests the possibility that the nitrogen atom, which is most readily removed by treatment with nitrous acid, is not the nitrogen which combines with the acid.

TABLE 6.—*Acid-combining capacities of deaminated gelatin and wool*

Material	Acid combined, per gram	Calculated acid-combining capacity of the guanidine nuclei
	Milliequivalents	*Milliequivalents*
Gelatin (23)	0.44	0.56
Wool (3)	.43	.52

V. CONCLUSION

The results of the investigation show that when wool, collagen, and arginine are treated with nitrous acid, increasing amounts of nitrogen are evolved with time. The continued evolution of nitrogen is due to the action of nitrous acid on the guanidine nuclei of these materials. Since the evidence indicates that the nitrogen which comes from the guanidine group is not free amino nitrogen, the amino-nitrogen contents of wool and collagen are calculated by correcting for the guanidine nitrogen evolved. The values obtained for the percentages of the total nitrogen as amino nitrogen are 2.53 for wool and 2.77 for collagen.

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